**RESEARCH ARTICLE**

## Mucosal Immune Responses against Live Newcastle Disease Vaccine in Immunosuppressed Chickens

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## ABSTRACT

To evaluate mucosal immunity of normal and immunosuppressed chickens vaccinated with live Newcastle disease (ND) vaccine, cyclophosphamide (CY) was used to generate immunosuppressed chickens. Normal and immunosuppressed chickens were vaccinated with the Lasota ND vaccine by ocular-nasal route at three weeks of age and challenged with virulent ND virus (vNDV) at day 28 post-vaccination (pv). The immunosuppressed chickens had significantly lower relative weight of the bursa of Fabricius and serum antibody HI titers compared to CY-untreated chickens. Compared with normal chickens, significant lower levels of IgA antibodies were detected in tracheal washings, duodenal washings and bile of immunosuppressed chickens in the whole experimental period. Immuno-histochemical experiment also showed that small numbers of IgA positive cells were found in intestinal tissues of immunosuppressed chickens at day 28 pv. There was only a partial protective effect on immunosuppressed chickens post challenge with virulent ND virus (vNDV). These findings increase our understanding of the protective mucosal immune response against ND vaccine and suggest that mucosal immunity play an important role against NDV infection.

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## INTRODUCTION

Immunosuppression is an abnormal immune state which leads to decrease the immune response to antigens and increase the sensitivity to diseases. The immunosuppression of chicken flock, caused by many factors such as infection and stress, has become a common biological phenomenon in poultry industry and caused severe economic losses. Immunosuppression has an obvious adverse effect on chicken flocks, which often causes secondary infection, multiple infections, or reduces immune effect of chicken flocks on commonly used vaccines (Sharma *et al*., 2000).

The immune system of chicken is different from that of mammal. The bursa of Fabricius is the unique central immune organ of chicken, which plays a critical role in the normal development of B lymphocytes in chickens (Weill and Reynaud, 1987). The bursa-dependent B-cell system in the chicken offers a good model to study ontogenetic development of B lymphocytes (Nowak *et al*.,

1990). Cyclophosphamide (CY) is a strong immuno-suppressant agent which can cause depletion of B lymphocytes and affect primarily antibody-mediated immunity. It has been reported that injection of CY to chickens induced selective B-cell damage to result in humoral immunosuppression (Corrier *et al*., 1991; Reynolds and Maraqa, 1999). So CY-induced immune-deficiency in newly hatched chickens provides a useful method to study the host defence mechanisms of humoral immunity following microbial infections (Linna *et al*., 1972; Markowski-Grimsrud and Schat, 2003).

Newcastle disease (ND) is one of the most important diseases affecting poultry throughout the world (Al-Garib *et al*., 2003; Alkhalaf, 2009; Shahzad *et al.*, 2011). Vaccine immunization is the major measure to prevent ND and has obtained good effect (van Boven *et al*., 2008). However, there are still immunity failures in poultry which have become the major problem in immune prevention of this disease. It is generally acknowledged that humoral immunity is the main immunity to Newcastle

disease virus (NDV). The immunity to NDV was most commonly evaluated by measuring antibody titer in the sera by hemagglutination inhibition (HI) test and high titer of antibodies were generally accepted as a reliable indicator of flock immunity (Beard and Hanson, 2003). However, the phenomenon that the flock was not protected after challenge with virulent NDV while there was high level antibody in serum or was protected while there was only very low level antibody in serum still occurred (Awan *et al.*, 1994). And there were ample evidences that HI antibody titer in serum were not directly correlated with the level of resistance of chickens to experimental NDV challenge (Jayawardane and Spradbrow, 1995; Reynolds and Maraqa, 2000; Erf, 2004). Although Cell-mediated immune response may be detected as early as two to three days after vaccination with an attenuated ND vaccine, it is not sufficient by itself to protect against virulent NDV (Reynolds and Maraqa, 2000). It has been proved that the mucosal immunity represented by IgA production plays an important role in the development of protection in chickens vaccinated against ND vaccine (Takada and Kida, 1996; Reynolds and Maraqa, 2000; Scott, 2004). The bursa of Fabricius is the central lymphoid organ for B-cell lymphopoiesis and lymphocyte maturation, which provides the micro-environment necessary for the rapid growth of B cells that have undergone the genetic changes necessary to produce effective antibodies against foreign antigens (Tizard, 2002; Ratcliffe, 2006). However, there are few reports about the effect of the damage or eccyliosis of bursa of Fabricius on local mucosal immune response in chickens vaccinated with live ND vaccine.

The aim of this experiment was to establish immunosuppressive model by treating SPF chickens with CY. Using this model, immunosuppression effect on mucosal immunity in SPF chickens vaccinated with live ND vaccine was characterized. Our results clearly showed that the local mucosal immune response played an important role in resistance to vNDV challenge.

## MATERIALS AND METHODS

### Chickens

Specific-pathogen-free (SPF) chickens (White Leghorn) were obtained from SPAFAS Bio-products Company in Shandong, China. During the experimental period, chickens were reared in separate units under positive pressure with filtered air. The chickens were provided with a starter commercial ration and potable water *ad libitum*.

### Vaccine and virus

The ND live vaccine (Lasota strain) was purchased from Qilu Animal Health Products Co. Ltd. (Shandong, China) and used for vaccination of experimental chickens at 3 weeks of age. One dose contained $10^6$ 50% embryo infection dose (EID$_{50}$) NDV. The virulent NDV $F_{48}E_9$ strain (vNDV) with the titer of $10^{7.35}$/0.1ml 50% embryo lethal dose (ELD$_{50}$) was purchased from China Institute of Veterinary Drugs Control and used for challenge experiment. The coated antigen in ELISA assay was prepared from NDV Lasota strain as previously described (Lambrecht *et al.*, 2004) and named inactivated NDV.

### Immunosuppression of chickens

CY (Sigma-Aldrich, St. Louis, MO, USA) was used as the regimen for immunosuppression of chickens. Within 24 h after hatching, SPF chickens were inoculated intramuscularly with 3 mg of CY per day for 4 consecutive days (Lam and Hao, 1987). The level of immunosuppression was evaluated by the relative weights of immune organs and the titers of serum antibody of experimental chickens.

### Experimental design and samples collection

150 one-day-old SPF chickens were used in this study. The chickens were randomly divided into 3 groups each with 50. Group 1 was neither treated with CY nor vaccinated with live ND vaccine and served as negative control group. Group 2 was untreated with CY and group 3 was treated with CY. At 3 weeks of age, each chicken of group 2 and 3 was vaccinated with one dose Lasota strain ND live vaccine by ocular-nasal route.

Firstly, three chickens from each group were sacrificed at 7, 14, 21, 28 and 35 days age. The bursa of Fabricius, thymus glands and spleens were weighed and the relative weight of each organ was calculated as a fraction of the carcass weight (g kg$^{-1}$ body weight). Secondly, three chickens from each group were sacrificed at days 3, 7, 14, 21, 28, 35 and 42 pv. Blood samples (3.0 ml per chicken) were drawn from the heart of chickens and allowed to clot at 37°C for 2 h prior to collect serum. Serum was separated by centrifugation and stored at -20°C before use. Tracheal washings were obtained by inserting a needle into the end of the trachea, then 5 ml of cold PBS containing 1% bovine serum albumin (BSA, 1% BSA/PBS) was slowly flushed in and out the tracheal lumen 10 times with a syringe. Duodenal washings were derived from 5 cm duodenum by lavage with 5 ml cold 1% BSA/PBS containing 0.1mg/ml soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Bile was collected from the gall bladder using a 22-gauge needle. All samples were stored at -20°C before subjecting to ELISA assay. For immunohistochemistry, samples of duodenum, jejunum and ileum were fixed in 4% paraformaldehyde.

### Measurement of the NDV specific humoral immunity

NDV specific humoral immunity was evaluated by HI test (Rauw *et al.*, 2009). In this experiment, four hemagglutination (HA) units of the Lasota strain of NDV were used. The HI was measured using a two-fold dilution series of the sera, and the titers were expressed as log$_2$ values of the highest dilution showing complete inhibition of agglutination.

### Measurement of the NDV specific local antibody-mediated immunity

Local antibody-mediated immunity to NDV was measured by NDV specific IgA ELISA as previously described (Rauw *et al.*, 2009). A checkerboard titration was performed to determine the optimum concentration of all reagents and carried out as follows: 96 microwell plates were coated overnight at 4°C with 15.7 µg/ml inactivated NDV diluted in 0.05 M carbonate/bicarbonate pH 9.6 buffer. The following day, plates were washed three times with PBS containing 0.05% Tween 20 (PBST)

and blocked with PBST containing 1% BSA (1% BSA/PBST) for 1h at 37°C. Tracheal washings, duodenal washings and bile from the experimental chickens were diluted in 1% BSA/PBST and added to the plate and then incubated for 1h at 37°C. After washing five times, HRP-conjugated goat anti-chicken IgA (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:10,000 in 1% BSA/PBST was added and incubated for 1 h at 37°C. After six washings, the plates were incubated with substrate solution tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C before stopping the reaction by adding 2 M $H_2SO_4$ solution. The optical density (OD) value of each well at 450 nm was read on a microplate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, USA).

**Immunohistochemical detection of IgA positive cells in chicken intestines**

For immunohistochemistry, samples of duodenum, jejunum and ileum were fixed in 4% paraformaldehyde and embedded in paraffin according to the conventional method. Serial paraffin sections, 5 µm thickness cut with a Leitz HM-500 cryostat were immunostained by Strep-tavidin-biotin-peroxidase (SP) method as described previously (Oliveira *et al*., 2006). In brief, after the slides were routinely deparaffinized, heat-induced antigen was retrieved by microwave pretreatment in citric acid buffer (10 mM; pH 6.0) for 20 min before staining. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methyl alcohol for 30 min followed by washing with PBS for 5 min. Non-specific binding was minimized by treating the sections with 5% normal goat serum in PBS for 30 min. The sections were then incubated in a humidified chamber overnight at 4°C with mouse anti-chicken IgA (1:100, Southern Biotech, USA). For negative control, 1% BSA was used instead of the primary antibody. After washed, slides were incubated for 30 min at room temperature with biotin-labeled secondary antibody working fluid (Vector Laboratories, USA). After washed, slides were incubated for 10 min at room temperature with avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Fresh diaminobenzidine (DAB, Maixin-Bio, Fuzhou, China) solution were applied for 10 min, the sections were counterstained with hematoxylin and mounted.

**Challenge experiments**

At day 28 pv, 20 chickens from each group were placed in an isolated animal unit and challenged with $10^4$ $ELD_{50}$ of vNDV in 0.2 ml PBS per chicken by ocular-nasal route. After challenge, clinical symptoms and mortality were monitored daily for 14 days. Chickens that showed the clinical typical signs of ND or died were considered as unprotected. This study was approved by Department of Science and Technology of Shandong Province. All animal experiments were performed according to national regulations and institutional guidelines.

**Statistical analysis**

Data were analyzed using the statistical software program SPSS18.0. The results were expressed as mean±standard deviation (mean±SD). Comparisons of means were conducted using Duncan's multiple range tests. P<0.05 was considered as statistically significant.

## RESULTS

**Effect of CY on relative weights of organs**

The relative weights of the bursa of Fabricius, thymus and spleen to the body weight of chickens were calculated at different days after treated with CY (Table 1). From 7 days to 35 days age, there was no significant (P>0.05) difference between the relative weights of immune organs of the control group and CY-untreated group. But the relative weights of the bursa of Fabricius of CY-treated group were significantly decreased (P<0.01) compared with the control group and CY-untreated group. And the relative weights of thymus and spleen of CY-treated group were significantly lower than that of CY-untreated group (P<0.05) from 7 days to 21 days age, however, the difference was not significant from 28 days to 35 days age (P>0.05).

**Serum HI antibody response to NDV**

Antibody titers of sera from experimental chickens pv were evaluated by HI test (Fig. 1). No HI antibody response to NDV was detected in the control group. In CY-untreated group HI antibody titres to live ND vaccine gradually rose from day 7 pv and peaked at day 21. However, in CY-treated group HI titres were significantly lower (P<0.01) than CY-untreated group throughout the experimental period.

**NDV specific IgA response in tracheal and duodenal washings and bile**

The levels of NDV specific IgA in tracheal washings (Fig. 2A), duodenal washings (Fig. 2B) and bile (Fig. 2C) of experimental chickens pv with ND live vaccine were evaluated by ELISA. The levels of IgA in tracheal washings of CY-untreated vaccinated chickens rose from day 3 pv and reached the maximum at day 28 pv However, the level of IgA detected in CY-treated group was statistically lower than that of the CY-untreated group (P<0.01) in the whole experimental period. At days 3 to 14 pv, the levels of IgA in tracheal washings of CY-treated group were very low, but gradually rose and reached the maximum at day 28, which was significantly higher than the control group (P<0.05). NDV specific IgA responses to ND vaccine in duodenal washings and bile were similar to that in tracheal washings.

**IgA positive cells in duodenum, jejunum and ileum**

Intestinal mucosal IgA positive cells were detected by immunohistochemical technique. The results showed that IgA positive cells were mostly located at the lamina propria, submucosa, and core of the villi of the different parts of the intestine, such as the duodenum (Fig. 3A), jejunum (Fig. 3B), and ileum (Fig. 3C). As showed in Fig. 3, CY-untreated chickens had significantly higher numbers of IgA positive cells in duodenum (Fig. 3D), jejunum (Fig. 3E) and ileum (Fig. 3F) than that of CY-treated chickens (Fig. 3G, H, I) at day 28 p.v.

**Table 1:** The relative weights of the bursa of Fabricius, thymus gland and spleen (g kg$^{-1}$ body weight) of chickens in the different experimental groups

| Organ | Group | Age (days) | | | | |
|---|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 | 35 |
| Bursa of | 1 | 2.21±0.25** | 3.56±0.22** | 4.64±0.18** | 4.45±0.17** | 4.53±0.19** |
| Fabricius | 2 | 1.77±0.23** | 3.76±0.21** | 3.92±0.10** | 4.67±0.40** | 4.55±0.20** |
| | 3 | 0.95±0.10 | 0.45±0.03 | 0.64±0.05 | 0.77±0.05 | 0.82±0.03 |
| Thymus | 1 | 4.88±0.12** | 5.10±0.37* | 6.29±0.17* | 5.18±0.32 | 5.53±0.21 |
| | 2 | 5.38±0.26** | 5.71±0.38* | 5.79±0.17* | 5.65±0.23 | 6.02±0.19 |
| | 3 | 2.69±0.25 | 4.15±0.37 | 4.26±0.17 | 5.05±0.47 | 5.27±0.24 |
| Spleen | 1 | 1.27±0.08* | 1.35±0.03* | 1.31±0.03* | 1.37±0.05 | 1.64±0.11 |
| | 2 | 1.34±0.14* | 1.26±0.04* | 1.31±0.05* | 1.32±0.04 | 1.62±0.05 |
| | 3 | 1.02±0.15 | 0.93±0.08 | 1.10±0.04 | 1.28±0.11 | 1.54±0.08 |

The group 1 was control, the group 2 was CY untreated and vaccinated, the group 3 was CY treated and vaccinated. Data are represented as mean ± SD (n=3) (*P<0.05, **P<0.01 by Duncan's multiple range test.).

**Table 2:** Relationship between pre-challenge serological results and post-challenge mortality

| Group | Pre-challenge | | | Post-challenge | |
|---|---|---|---|---|---|
| | Tracheal washings IgA (OD$_{450}$) | Duodenal Washings IgA (OD$_{450}$) | Serum HI Titers (log$_2$) | Survivors (%) | Serum HI Titers (log$_2$) |
| 1 | 0.080±0.008[a] | 0.093±0.012[a] | 0±0.0[a] | 0[a] | 5.5±0.3[a] |
| 2 | 0.823±0.023[b] | 0.988±0.026[b] | 6.0±0.3[b] | 100[b] | 7.5±0.4[b] |
| 3 | 0.418±0.019[c] | 0.452±0.015[c] | 1.0±0.1[c] | 60[c] | 1.5±0.1[c] |

The group 1 was control, the group 2 was CY untreated and vaccinated, the group 3 was CY treated and vaccinated. Data are presented as mean ± SD. Means in the same row with different superscript a, b and c is significantly different (P<0.05).
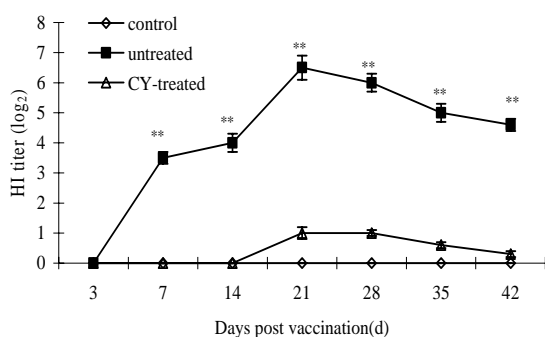


**Fig. 1:** The titer of specific serum antibodies to NDV in the different experimental groups. Antibody titers were subjected to log$_2$ transformation. Data are represented as mean ± SD at each time point (n=3) (**P＜0.01 by Duncan's multiple range test).

**Relationship between pre-challenge serological results and post-challenge mortality**

As shown in Table 2, after challenged with vNDV, the chickens of control group showed the first clinical signs at day 3, and all died by day 6. Prior to challenge, the level of IgA in tracheal and duodenal washings of CY-untreated group was significantly higher than control group (P<0.05), and the serum HI titer was $2^{6.0}$, thus all 20 chickens survived post challenge. The immune protective rate was 100%. However, before challenge, serum HI titer of CY-treated group was only $2^{1.0}$ which was significantly lower than CY-untreated group (P<0.05), while the local IgA level in tracheal and duodenal washings was significantly higher (P<0.05) than control group. Finally there were 12 chickens survived post challenge and the immune protective rate was 60%. The results fully indicated that local IgA antibody in respiratory and digestive tract played an important role in resistance to vNDV challenge.

## DISCUSSION

Bursa of Fabricius, thymus and spleen are important immune organs of poultry. Thymus and bursa of Fabricius are the sites where T and B lymphocytes differentiate, develop and mature. Spleen is the important site where immune response occurs (Niu, 2004). So the immune level of poultry is closely correlated with the development and function of these organs (Liu *et al*., 2006). The relative weight of immune organ to body weight is a commonly used index to reflect the development status of immune organs to some extent (Dong *et al*., 2007). CY is a common immunosuppressant which can cause marrow hematopoiesis suppression and immunosuppression. CY can suppress B and T lymphocytes and is especially sensitive to B lymphocytes. The effects of CY were generally regarded as a form of chemical bursectomy (Elmubarak *et al*., 1981). It was reported that CY could suppress the development of bursa of Fabricius of chicken and the proliferation of B lymphocytes as well as reduce humoral immunity (Hemendinger and Bloom, 1996). Glick (1986) also reported that injection of CY could primarily suppress bursa-dependent B lymphocytes and suppress humoral immunity. Injection of CY could eliminate the humoral immune response to infectious bursal disease virus (IBDV) of young chicken, suppress the response to ConA of lymphocytes and significantly reduce the amount of peripheral blood B lymphocytes (Yeh *et al*., 2002).

In the present study, we treated experimental chickens by CY as described previously (Lam and Hao, 1987) and established immunosuppressive model. The results indicated that CY-treated chickens had significantly lower relative weights of the bursa of Fabricius compared with CY-untreated group during 35 days after treatment (P<0.01). However, the relative weights of thymus and spleen in CY-treated group were lower than CY-untreated group only in early period (7-21d age) (P<0.05) (Table 1). The result was consistent
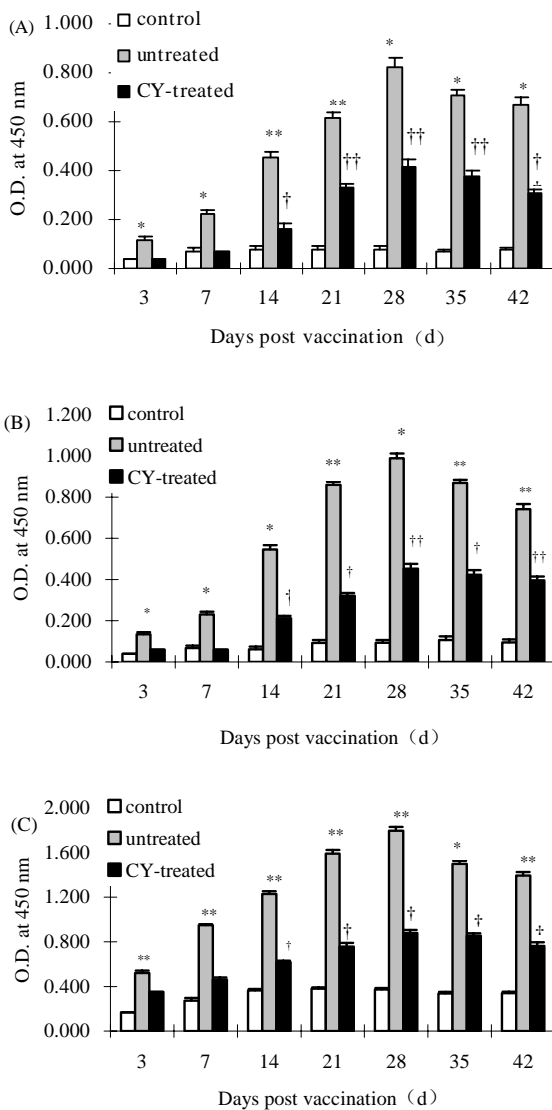
**Fig. 2:** NDV specific IgA response to ND vaccine in tracheal washings (A), duodenal washings (B) and bile (C). Bars represent mean±SD of OD values at 450 nm were determined by ELISA at specified time pv ($n$ = 3). Tracheal washing samples were diluted at 1:5. Duodenal washing samples were diluted at 1:50. Bile samples were diluted at 1:50. Significant differences between untreated group and CY-treated group are expressed as *P<0.05 and **P<0.01, between CY-treated group and control group as †P<0.05 and ††P<0.01 by Duncan's multiple range test.

with those reported by Corrier *et al.* (1991), Reynolds and Maraqa (1999) and Ei-Abasy *et al.* (2004). It had been reported that the treatment of chickens with CY resulted in a severe and significant suppression on humoral immunity and significantly reduced serum antibody HI titer p.v. with ND vaccine (Eskola and Toivane, 1974; Lam and Hao, 1987). Our data confirmed the previous reports. At day 21 p.v., serum antibody HI titer of CY-treated group was extremely significant lower than that of CY-untreated group (P<0.01). These results fully demonstrated that the immunosuppressive model of chickens by CY was successfully mimicked.

On one hand, the humoral immunity was suppressed by CY. On the other hand, stem cells of B lymphocytes could not differentiate and mature in the bursa of Fabricius, let alone migrate to peripheral lymphoid tissues because the bursa of Fabricius could not normally develop and severely atrophy. As a result, bursa-department B lymphocytes in peripheral lymphoid tissues (organs) might significantly decrease. In the present study, levels of specific IgA in tracheal washings, duodenal washings and bile of CY-treated group were significantly lower than that of CY-untreated group (P<0.05) (Fig. 2). In addition, the results of immunohistochemistry showed that the population of IgA positive cells in intestinal mucosa of CY-treated group were significantly less than CY-untreated group at day 28 pv (Fig. 3). These results indicated that precursor cells of IgA positive cells in peripheral lymphoid tissues might be bursa-dependent cells and mostly originate from lymphoid stem cells differentiating and maturing in the early bursa. If the bursa of Fabricius was suppressed, local mucosal immune response was also suppressed. A common truth was indicated that systemic humoral immunity and local mucosal immunity should be correlated with each other and not absolutely independent from each other.

However, the suppression of CY is not indefinite and everlasting. At the late stage of experiment, although the bursa of Fabricius of chickens is damaged, the hemopoiesis of bone marrow may generally recover. Thus new B lymphocytes originating from bone marrow can flow into and plant in peripheral lymphoid organs and mucosa associated lymphoid tissues (MALT). In addition, local germinal centers of B lymphocytes may gradually form in MALT. So the suppressed effect on mucosal immune response in local mucosa by CY may recover during the late stage of development. As expected, levels of IgA in tracheal washings, duodenal washings and bile of CY-treated group significantly rose from day 14 pv (Fig. 2). And a certain amount of IgA positive cells were also found in intestinal mucosa of CY-treated group at day 28 pv (Fig. 3). In addition, 60% chickens of CY-treated group survived the vNDV challenge (Table 2). Previous studies proved that CY treatment in the newly-hatched period had little effect on the thymus and left cellular immunological capacities intact (Linna *et al.*, 1972; Kowalski *et al.*, 1978). So the cellular immunity of CY-treated chickens might mostly recover at day 28 pv and was not significantly different from untreated birds. The above results fully proved that local mucosal immune response played an important role against NDV infection.

In conclusion, the present study firstly demonstrated that mucosal immune response of chickens vaccinated with live ND vaccine was significantly impaired by CY treatment after hatching. And we also found that the immunosuppressed effect on mucosal immunity by CY was partly recovered from day 21 pv. In addition, the direct evidences were obtained for the role of mucosal immunity in resistance to vNDV by challenge experiment. These findings provide a better understanding of the protective mucosal immune response against ND vaccine and suggest that mucosal immunity play an important role against NDV infection.
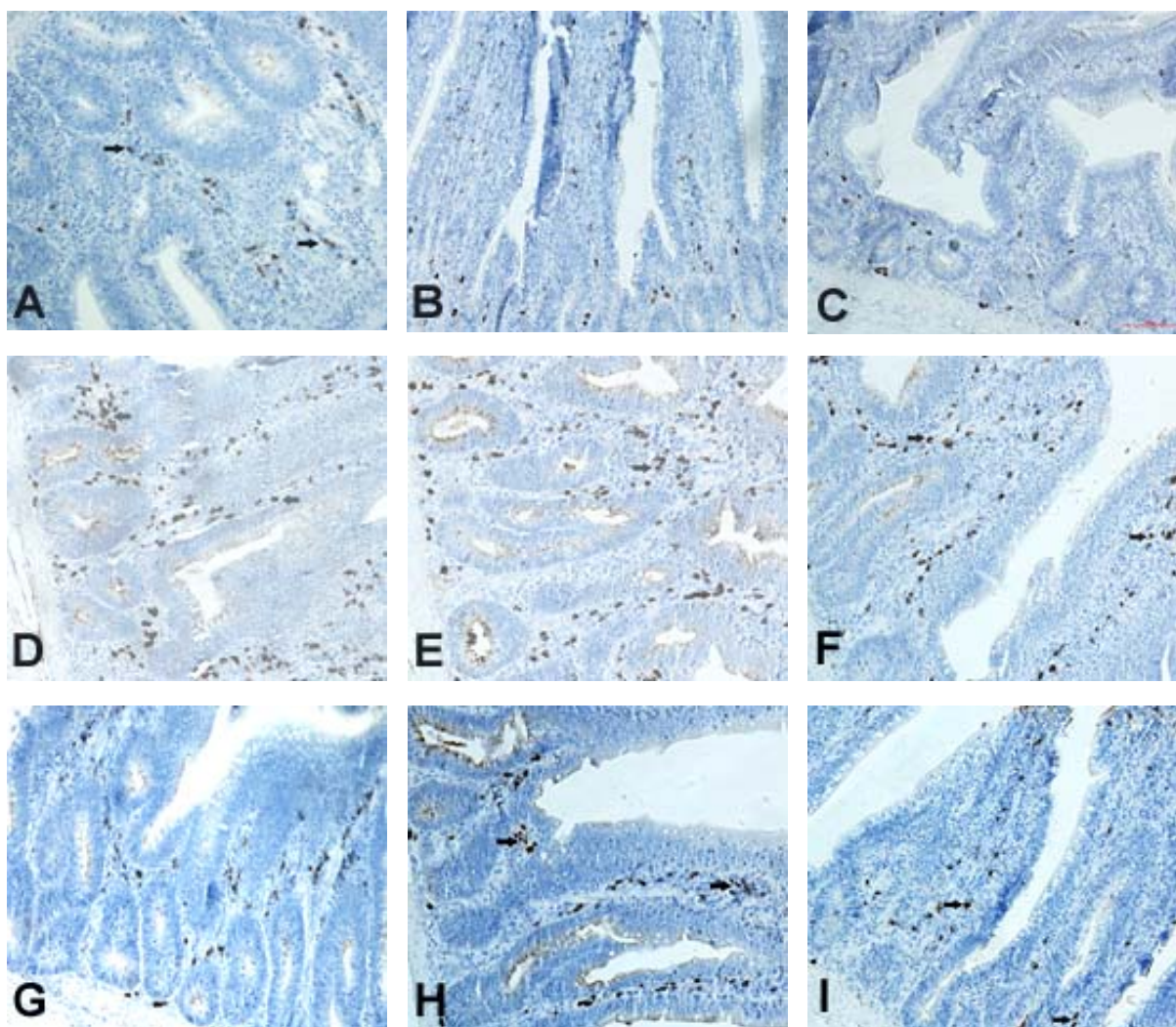
**Fig. 3:** At day 28 pv with ND vaccine, a large number of IgA positive cells (arrow) could be seen in the lamina propria of duodenum (D), jejunum (E) and ileum (F) in untreated group. A small number of IgA positive cells (arrow) were also present in the lamina propria of duodenum (G), jejunum (H) and ileum (I) in CY-treated group. Occasional IgA positive cells were detected in the lamina propria of control duodenum (A), jejunum (B) and ileum (C) (×200).

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